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γ-Glutamyltransferase of Rat Kidney

SIMULTANEOUS ASSAY OF THE HYDROLYSIS AND TRANSFER REACTIONS WITH [*GLUTAMATE*-^{14}C]GLUTATHIONE

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1. The hydrolytic and transfer reactions catalysed by rat kidney γ-glutamyltransferase (EC 2.3.2.2) were studied *in vitro* with the substrates [U-^{14}C]glutamic acid-labelled glutathione and methionine. Initial-velocity patterns, isotope-exchange and binding studies were consistent with a branched non-sequential mechanism in which a γ-glutamyl-enzyme intermediate may react either with water (hydrolysis) or with methionine (γ-glutamyl transfer). 2. The Michaelis constant for glutathione in hydrolysis was $13.9 \pm 1.4\,\mu$M, for glutathione in transfer it was $113 \pm 15\,\mu$M and for methionine as substrate it was 4.7 ± 0.7 mM. At substrate concentrations in the ranges of their respective Michaelis constants, the rate of transfer was about ten times higher than that of hydrolysis, but at concentrations of methionine approximating to the physiological ($64\,\mu$M in rat plasma) the transfer is negligible. 3. The enzyme is reported to lie on the luminal surface of the proximal straight kidney tubule. In this situation, if the kinetic results obtained with the detergent-solubilized enzyme are relevant to the behaviour of the enzyme *in vivo*, it appears likely that the main function of renal γ-glutamyltransferase is not in amino acid transport, but rather to hydrolyse glutathione in the renal filtrate.

It has usually been stated that a major pathway of catabolism of GSH involves the enzyme γ-glutamyltransferase (EC 2.3.2.2),* but the nature of the reactions catalysed by this enzyme and even its substrates *in vivo* are not known with certainty, so that its physiological role is not entirely clear. *In vitro*, the enzyme is known to catalyse the hydrolysis of the γ-glutamyl groups of many compounds, including those of GSH and glutamine, and also the transfer of the γ-glutamyl group to a variety of acceptors (Orlowski & Meister, 1970; Meister, 1973). Owing largely to the difficulties of assaying the hydrolysis at low concentrations of GSH, it is the transfer reaction that has received most attention. The relative importance of hydrolysis and transfer, and Michaelis constants for many of the substrates, have not been determined. It has been shown in the rat kidney that γ-glutamyltransferase is identical with a previously recognized phosphate-independent glutaminase (Curthoys & Kuhlenschmidt, 1975). The enzyme is located on the external surface of the brush-border membranes, apparently of the proximal straight tubule (Kuhlenschmidt & Curthoys, 1975), although previous reports indicated the proximal convoluted tubule (Meister, 1973). Of the two most obviously probable substrates, the concentration of GSH in the tubular filtrate is probably not greater than about $30\,\mu$M (Elwyn, 1966; Waelsch, 1952; Elwyn *et al.*, 1968); the concentration of glutamine in the proximal straight tubule is not known, but it is usually assumed that amino acid re-absorption is essentially complete in the proximal convoluted tubule. Although the enzyme is probably also implicated in the mercapturic acid pathway of drug metabolism (Elce *et al.*, 1974), it seems improbable that this should be its fundamental physiological role. Much evidence, largely from Meister's laboratory, suggests that the enzyme may be involved in amino acid transport in the renal tubule and elsewhere (Meister, 1974). On the other hand, some of this evidence is hard to reconcile with conventional views of amino acid transport in the kidney (Milne, 1971).

Some of the confusion arises from inadequate assay methods for γ-glutamyltransferase, since the existing assays require GSH concentrations in the mM range or use artificial substrates, and give no measure of hydrolysis. It was therefore important to obtain more precise information about the mechanism of action of the enzyme, the relative contributions of hydrolysis and transfer and the values of the Michaelis constants of the more probable substrates. An assay for the transfer reaction using the natural substrate GSH and [^{14}C]methionine,

* The Enzyme Commission name for this enzyme is γ-glutamyltransferase, but a great deal of the recent literature uses the name γ-glutamyltranspeptidase. It seems likely that neither name is a reflection of its physiological role.

at concentrations down to 5 μM, was therefore developed in this laboratory (Elce *et al.*, 1973, 1974). Although the method gave some estimate of the Michaelis constants, it became clear that for further progress a GSH substrate molecule carrying a radioactive label in the glutamic acid residue was essential. This labelled GSH is not commercially available, and the reported methods for obtaining it, by means of exchange processes in erythrocyte lysates (Hochberg & Dimant, 1965) or by the use of glutathione-synthesizing enzymes (Mooz & Meister, 1971), are incapable of providing the necessary quantity, purity and specific activity. [U-^{14}C]Glutamic acid-labelled glutathione ([^{14}C]GSH) was synthesized for us by New England Nuclear Corp. (Boston, Mass., U.S.A.), who supplied 0.5 mCi with a specific activity of 16.6 mCi/mmol. With this substrate it became possible for the first time to observe precisely the metabolism of the γ-glutamyl residue, and to measure simultaneously both the hydrolytic and the transfer reactions.

Experimental

Materials

The materials and methods used were largely the same as those previously described (Elce *et al.*, 1974). [U-^{14}C]Glutamic acid-labelled glutathione (16.6 mCi/mmol) was diluted with non-radioactive GSH to specific radioactivities of 5.0 mCi/mmol and 1.0 mCi/mmol. In view of the mode of chemical synthesis of the [^{14}C]GSH, it was certain that the ^{14}C was present only in the glutamic acid residue, but it was necessary to check the chemical composition and specific radioactivity of the sample in order to obtain precise kinetic results. The radioactivity observed in 1 μmol of the 1.0 mCi/mmol stock solution was 0.986 μCi, and, following performic acid treatment and 6 M-HCl hydrolysis, 1 μmol of [^{14}C]GSH was shown to contain 1.0 μmol of glutamic acid, 1.09 μmol of cysteine and 0.93 μmol of glycine. L-[4-^{3}H]Glutamic acid was obtained from Schwarz Bioresearch, Orangeburg, N.Y., U.S.A. γ-Glutamyl[*Me*-^{3}H]methionine was prepared as previously described (Elce *et al.*, 1974). Performic acid was prepared by mixing 9 ml of 90% (w/w) formic acid with 1 ml of 30% (w/w) H_2O_2 solution, and leaving the mixture for 1–2 h before use (Hirs, 1967).

Enzyme preparation

The enzyme was obtained from kidneys of adult male hooded rats (Charles River; Long Evans, outbred strain; 250–300 g) by a slight modification of a published procedure (Elce *et al.*, 1974). The process involves sodium deoxycholate treatment of a microsomal pellet (106000 $g_{av.}$, 90 min) from 20 rat kidneys, followed by chromatography on a column (70 cm × 2.5 cm) of DEAE-cellulose (200 g) in the presence of 0.1% Triton X-100. The fractions from the DEAE-cellulose column which contained the highest specific activity of the enzyme were dialysed against buffer without Triton X-100, and the enzyme solution was applied to a column (42 cm × 2.5 cm) of Sephadex G-200 (7 g; Pharmacia, Dorval, P.Q., Canada) packed in 0.01 M-Tris/HCl/0.15 M-NaCl, pH 8.5, at 4°C (not containing Triton X-100), and eluted with the same buffer. The main peak of enzyme activity was eluted at the void volume of the column just before the main protein peak. This preparation was stored at 4°C and has been used for about 1 year without detectable loss of activity. For most kinetic assays a sample of the enzyme preparation was diluted with 9 vol. of the incubation buffer, and 10 μl of the diluted preparation was used for each incubation. This volume contained approx. 0.125 μg of protein and 1.5×10^{-3} unit of γ-glutamylnaphthylamide activity (as defined by Elce *et al.*, 1974), which is about one-eighth of the amount of enzyme used previously (Elce *et al.*, 1974).

Initial-velocity studies

Each tube contained [^{14}C]GSH (5 μCi/μmol for the range 5–200 μM; 1 μCi/μmol for the range 0.1–1.0 mM), dithiothreitol (2 mM), magnesium acetate (5 mM), methionine (0–20 mM) and enzyme sample, in a total volume of 0.2 ml of 0.01 M-Tris/HCl, pH 8.5, at 37°C. After preincubation at 37°C, reaction was initiated by addition of the enzyme preparation stored on ice, and terminated after a suitable time-interval by addition of 0.2 ml of performic acid. When the hydrolysis was being studied, the 0.2 ml of performic acid also contained [^{3}H]glutamic acid (50 μg, approx. 60000 d.p.m.); for studies of the transfer, γ-glutamyl-[^{3}H]methionine (25 μg, approx. 30000 d.p.m.) was added immediately after the performic acid. Both ^{3}H-recovery tracers could be added to the same incubation tube if necessary. The mixtures with the performic acid were left at 4°C overnight, then 0.5 ml of ethanol was added to each tube and the contents were evaporated to dryness under N_2. The residues were transferred to Whatman 3MM paper sheets, which were subjected to electrophoresis in acetic acid/pyridine/water, (10:3:500, by vol.), pH 3.9, under Varsol (an insulating coolant fluid, obtained from Imperial Oil Ltd., Kingston, Ont., Canada) for 70 min at 3 kV. In this time glutamic acid migrated about 12 cm towards the anode, γ-glutamylmethionine sulphone about 27 cm and GSH sulphonic acid about 42 cm. After the Varsol and buffer had evaporated, the relevant sections of the sheets were sprayed with 0.2 M-$Na_2B_4O_7$/NaOH, pH 10.6, until very wet, and then dipped rapidly through a solution of 2 mg of fluorescamine in 50 ml of dry acetone

(Stein *et al.*, 1974). The light-green fluorescent areas corresponding to glutamic acid and γ-glutamylmethionine sulphone were cut out and chopped up into 1.5ml of 0.5M-acetic acid in scintillation vials. After 1h, 10ml of Aquasol (New England Nuclear Corp.) were added to each vial and the ^{3}H and ^{14}C radioactivity determined by liquid-scintillation counting. The recovery of ^{3}H to the counting vial from the sample added at the end of the incubation (usually about 65%) permits correction for losses of ^{14}C during performic acid oxidation, electrophoresis and elution.

Synthesis of γ-glutamyl-GSH sulphonic acid, and measurement of γ-glutamyl-GSH formation

The sulphonic acid of γ-glutamyl-GSH was prepared as follows, by using the method of Orlowski & Szewczuk (1962). GSSG (1mmol) was mixed well with phthaloyl-L-glutamyl anhydride (2.1mmol) and heated in suspension in 2ml of acetic acid at 63°C for 35min, forming a clear viscous gum, which was dried overnight under vacuum in the presence of solid $CaCl_2$ and NaOH. The gum was dissolved in 10ml of 0.5M-Na_2CO_3 and titrated to pH10 with 5M-NaOH; 2ml of 2M-hydrazine was added and the mixture left for 2 days. The pH was adjusted to 4 with 5M-HCl; this caused a white precipitate to form which was removed by filtration. The solution was diluted to 50ml with water and poured on to a column (12cm×2cm) of Dowex 1 X2 (acetate form), followed by stepwise elution with water (100ml), 1M-acetic acid (100ml) and 3M-acetic acid (100ml). As indicated by electrophoresis, the 1M-acetic acid eluted large amounts of unchanged GSSG; the 3M-acetic acid eluate contained mainly a compound which was found to be the mixed disulphide of γ-glutamyl-GSH and GSH. Some of this material was treated with performic acid and the product applied to a column (17cm×1cm) of DEAE-cellulose (Cl^{-} form) (Baugh *et al.*, 1974). The column was eluted with a linear gradient of 350ml of 0.005M-sodium potassium phosphate buffer, pH7.0, and 350ml of 1M-NaCl in the same buffer; 10ml fractions were collected, portions of which were assayed by the ninhydrin reaction. γ-Glutamyl-GSH sulphonic acid was eluted in fraction 10 and after hydrolysis with 6M-HCl was shown to contain cysteic acid, glutamic acid and glycine in the molar proportions 1.00:2.07:1.09.

It was necessary to determine the extent of γ-glutamyl-GSH formation which occurs if GSH acts as acceptor as well as donor of the γ-glutamyl group. Since the corresponding sulphonic acids were not separated by electrophoresis in the conditions used in the present work, γ-glutamyl-GSH sulphonic acid was determined by DEAE-cellulose chromatography, following addition of non-radioactive carrier (the mixed disulphide of γ-glutamyl-GSH and GSH) to incubation mixtures immediately after the performic acid. The fractions eluted from the column were assayed for radioactivity and ninhydrin response. The specific radioactivity of enzymically formed γ-glutamyl-GSH is twice that of the substrate [^{14}C]GSH.

Isotope exchange

Incubations of [^{14}C]methionine with γ-glutamylmethionine and the subsequent electrophoresis were performed precisely as described previously (Elce *et al.*, 1974).

Treatment of kinetic data

The experimental observations were the values of ^{14}C radioactivity (d.p.m.) of product formed in the 0.2ml incubation volume in a given time, corrected for procedural losses via the recovery of ^{3}H radioactivity (d.p.m.), but the observations were converted into μmol of product formed/min per litre. (These units, of change in micro-molarity of product per min, are desirable for computer processing of the data, since the rate equation parameters then emerge from the computer with units of molarity.) In those cases where it seemed likely to be fruitful, the data were fitted in the non-reciprocal form, without weighting, to appropriate rate equations (see Appendix) by non-linear regression on an IBM 360 computer (Cleland, 1967; Hurst *et al.*, 1973). The program supplied values of the kinetic constants with their standard deviations, and the sum of the squares of the residual errors. For purposes of illustration the data from some experiments are shown in the form of double-reciprocal plots; the straight lines on these plots were drawn from the predicted values of the experimental points supplied by the computer program.

Results

Hydrolysis

The initial rate of the hydrolysis reaction was measured as the rate of release of free [^{14}C]glutamic acid from [^{14}C]GSH. The rate of hydrolysis was proportional to the amount of enzyme used, up to at least 40μl of the diluted enzyme preparation; unless otherwise stated, 10μl were used. The results of a typical experiment in the absence of methionine in which the [^{14}C]GSH concentration varied from 3.4 to 42.9μM are shown in Fig. 1. The regression of these data with eqn. (1) of the Appendix gave values for $V_{max.h}$ of 0.59 ± 0.03μmol/min per litre and for K_{ah} of 13.9 ± 1.4μM. In the range 0.1–1.1mM, GSH did not appear to inhibit its own hydrolysis.

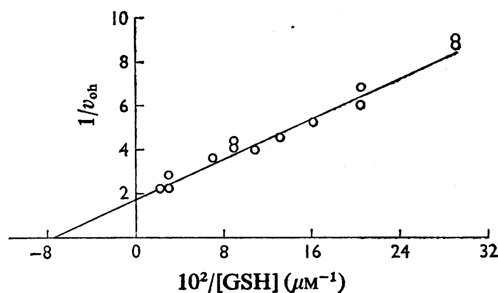

Fig. 1. *Hydrolysis of* [^{14}*C*]*GSH in the absence of methionine*

The incubation mixtures contained [^{14}C]GSH (5μCi/μmol), dithiothreitol (0.4mM), magnesium acetate (5mM) and 10μl of enzyme preparation (0.125μg of protein) in a final volume of 0.2ml of 0.01M-Tris/HCl, pH8.5. Three observations were made at each GSH concentration but are not all shown where they were very close together. The time of incubation varied from 2 to 10min. The initial velocities were expressed in μmol/min per litre, and the line was drawn from the computer analysis of the data with eqn. (1) of the Appendix.

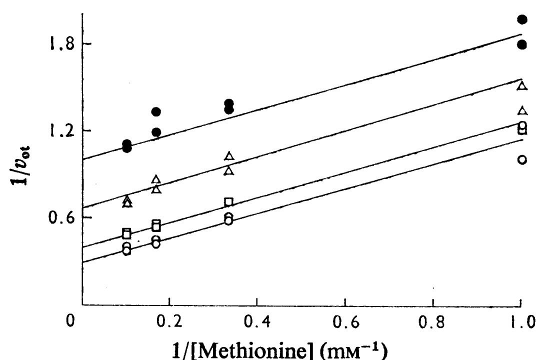

Fig. 2. *Formation of γ-glutamyl*[^{14}*C*]*methionine* (*transfer*)

The incubation mixtures contained [^{14}C]GSH (5μCi/μmol) at concentrations of 25.7μM (●), 40.9μM (△), 97.2μM (□) and 188.1μM (○), methionine, dithiothreitol (0.4mM), magnesium acetate (5mM) and 10μl of enzyme preparation (0.125μg of protein) in a final volume of 0.2ml of 0.01M-Tris/HCl, pH8.5. The time of incubation was 4min. The initial velocities were expressed in μmol/min per litre, and the lines were derived from the computer regression of the data with eqn. (3) of the Appendix.

Methionine acted as an inhibitor of the hydrolysis reaction. At concentrations of methionine greater than 10mM the hydrolysis was so slow that it could no longer be measured reliably. The data in a number of experiments in various substrate ranges did not distinguish statistically between competitive and non-competitive (eqn. 2 of the Appendix) inhibition patterns, and therefore could not be used to evaluate K_b.

Transfer

The initial rate of transfer was measured as the rate of formation of γ-[^{14}C]glutamylmethionine. The data obtained in several substrate ranges could not distinguish between non-sequential and sequential mechanisms. The results of an experiment in which the concentration of [^{14}C]GSH ranged from 25–188μM and that of methionine from 1.0–10.0mM are shown in Fig. 2. The regression analysis of these data, by using eqn. (3) of the Appendix, gave the following values: $V_{max.t} = 5.46 \pm 0.42\,\mu$mol/min per litre; $K_b = 4.67 \pm 0.7$mM; $K_{at} = 113 \pm 15\,\mu$M. The regression assigned a value not significantly different from zero to K_{ah} in eqn. (3), although a value of 13.9μM had been obtained previously from the hydrolysis data (see above). The presence of the $K_{ah} \cdot K_b$ term in the denominator of eqn. (3) distinguishes it from the corresponding equation for a simple non-sequential mechanism, and these two values for K_{ah}, 13.9μM and apparently zero, illustrate the difficulty of defining terms which are very small in relation to other considerably larger parameters in the rate equation. There is no doubt that a K_{ah} term is required and has a value in the region of 14μM, but the data were not sufficiently precise to confirm or deny the presence of the $K_{ah} \cdot K_b$ term in eqn. (3) of the Appendix, and therefore also could not distinguish sequential from non-sequential mechanisms.

When the [^{14}C]GSH concentration was greater than 0.5mM, the transfer was inhibited; the double-reciprocal plots (Fig. 3) were characteristic of substrate inhibition.

It was shown previously (Elce *et al.*, 1974) that cysteinylglycine acted as a competitive inhibitor of the transfer with respect to methionine, which is in accordance with the general form of eqn. (4) of the Appendix.

An experiment was performed to determine whether γ-glutamylmethionine formed by the transfer was significantly active as an alternative substrate of the enzyme during the normal incubation period. The rate of disappearance of γ-glutamyl[^{3}H]methionine (0.25mM) was measured in incubation tubes which also contained non-radioactive GSH (0.1mM), methionine (5mM) and 10μl of enzyme preparation. No loss of ^{3}H from the γ-glutamylmethionine area could be detected over periods of up to 30min. In the absence of added γ-glutamylmethionine, the concentration of this compound formed in these incubation conditions would be about 0.05mM.

Formation of γ-glutamyl-GSH

The data in Table 1 indicate that the amount of radioactivity in the γ-glutamyl-GSH sulphonic acid peak fractions, minus the control value, was about 8% of the initial radioactivity. Since the specific radioactivity of this product is twice that of the [14C]GSH, it appears that the maximum extent of formation of γ-glutamyl-GSH was of the order of 4%, starting from an initial [14C]GSH concentration of 4mM. It is not known why such a high value was observed in the control glutamic acid peak fraction on this occasion.

Isotope exchange

Mixtures of γ-glutamylmethionine (0.02–1.0mM) and [14C]methionine (0.057–0.51 mM) were incubated with approx. eight times the normal amount of enzyme used in the present work, and the rate of formation of γ-glutamyl[14C]methionine was measured, omitting the performic acid oxidation. The results of one such experiment are shown in Fig. 4. The derived values (eqn. 5 of the Appendix) were: $V_2^* = 27.7 \pm 7.1$ μmol/min per litre; $K_{ib} = 2.25 \pm 0.67$mM; $K_{iq} = 0.24 \pm 0.08$mM; $K_{qh} = 18.2 \pm 4.2$μM.

Variation of the rates of hydrolysis and transfer with pH

The variation of the initial rates was measured in phosphate buffer in the pH range 6–7 and in Tris/HCl buffer in the pH range 7.3–8.8. The rate of hydrolysis of 50μM-[14C]GSH in the absence of methionine and of 1.8mM-[14C]GSH in the presence of 2mM-methionine was approximately constant from pH 6 to 7, and decreased by about 40% over the pH range 7–8.8. The transfer was almost twice as fast as the hydrolysis at pH 8.8 (cf. Fig. 5) and decreased linearly to a negligible value at pH 7.0.

Nature of the reaction at possible physiological concentrations

The initial velocities of hydrolysis and transfer at [14C]GSH concentrations of 0.2 and 2.0mM over a range of methionine concentrations are shown in Fig. 5. It should be borne in mind that the reported concentration of GSH in rat kidney is 2.4mM (Davidson & Hird, 1964) and of methionine in rat plasma is 64μM (Seta *et al.*, 1973). The curves are very similar to one obtained by Goore & Thompson (1967) with the enzyme in the fruit of the kidney bean.

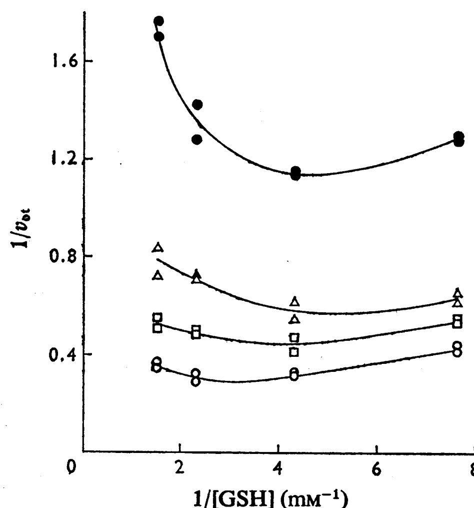

Fig. 3. *Inhibition of transfer by high concentrations of [14C]GSH*

The incubation mixtures contained methionine at concentrations of 1.0mM (●), 3.0mM (△), 6.0mM (□) and 12.0mM (○), [14C]GSH (5μCi/μmol), dithiothreitol (2mM), magnesium acetate (5mM) and 10μl of enzyme preparation (0.125μg of protein) in a final volume of 0.2ml of 0.01M-Tris/HCl, pH 8.5. The time of incubation varied from 4 to 20min. The initial velocities were expressed in μmol/min per litre, and the lines were drawn by eye.

Table 1. *Formation of γ-glutamyl-GSH*

[14C]GSH (4mM) was incubated in the usual system with an enzyme sample containing 25μg of protein for 0, 10 and 30min. The reactions were stopped with 0.2ml of performic acid containing [3H]glutamic acid, and about 100μg of the mixed disulphide of γ-glutamyl-GSH and GSH were added to each incubation mixture. After 24h the samples were evaporated to dryness and the residues chromatographed on DEAE-cellulose (Baugh *et al.*, 1974).

	[14C]GSH radioactivity (% of initial value)		
Time of incubation (min) Peak ...	Glutamic acid	GSH sulphonic acid	γ-Glutamyl-GSH sulphonic acid
0	9.9	79.2	3.7
10	36.2	51.7	11.6
30	76.0	21.6	3.9

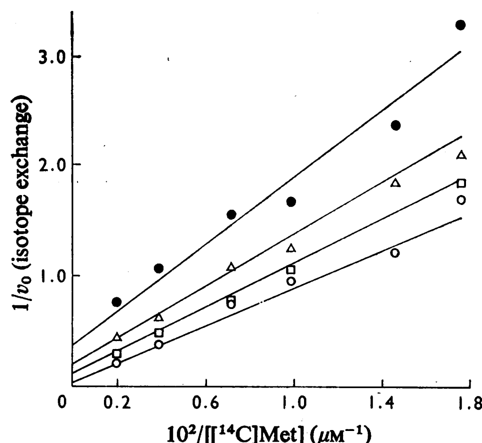

Fig. 4. *Isotope exchange between γ-glutamylmethionine and [^{14}C]methionine*

The incubation mixtures contained γ-glutamylmethionine at concentrations of 25μM (●), 50μM (△), 100μM (□) and 1000μM (○), [^{14}C]methionine (11.2mCi/mmol), dithiothreitol (0.4mM), magnesium acetate (5mM) and 10μl of a more concentrated enzyme preparation (Elce *et al.*, 1974) in a final volume of 0.2ml of 0.01M-Tris/HCl, pH 8.5. The time of incubation was 2min. The initial velocities were expressed in μmol/min per litre and the lines were drawn from the computer regression of the data with eqn. (5) of the Appendix.

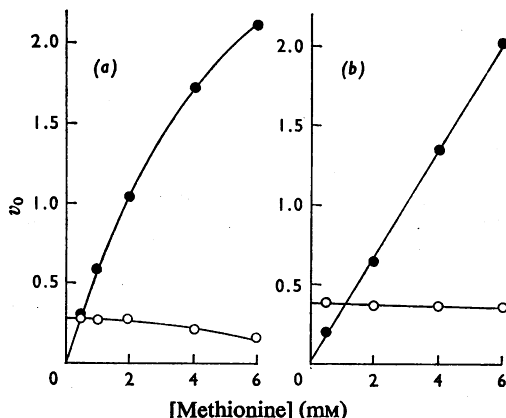

Fig. 5. *Relative contributions of hydrolysis and transfer*

The incubation mixtures contained [^{14}C]GSH at concentrations of (*a*) 0.2mM or (*b*) 2.0mM, together with methionine, dithiothreitol (2mM), magnesium acetate (5mM) and 10μl of enzyme preparation (0.125μg of protein) in a final volume of 0.2ml of 0.1M-Tris/HCl, pH 8.5. The time of incubation varied from 8 to 60min. The hydrolysis (○) and the transfer (●) were both measured in each incubation.

Detection of a γ-glutamyl-enzyme intermediate

The results in Table 2, obtained with a mixture of [^{14}C]GSH and [*Gly*-^{3}H]GSH, and very large amounts of enzyme, show that ^{14}C was more efficiently bound to the protein in the enzyme preparation than was ^{3}H. A plot of ^{14}C radioactivity (d.p.m.) in Table 2 (experimental-minus-control value) against protein is a straight line. Other control experiments showed that less than 20% of the substrate had reacted during the 10s incubation time used in the binding experiment.

Discussion

Comments on the method

If the performic acid oxidation was omitted, very large amounts of radioactivity were observed all along the electrophoretic track, and the peak of [^{14}C]GSH coincided with that of γ-glutamylmethionine over a wide range of pH values of the electrophoresis buffer. After performic acid oxidation, the products were well separated from each other and from [^{14}C]GSH sulphonic acid. The control values in each product area were 1.0±0.2% of the total ^{14}C radioactivity (d.p.m.) added to each incubation and were not time-dependent. Because of this, and to preserve linearity of the initial velocities, the times of incubation were varied widely, from 2 to 60min, to achieve about 10% reaction in every tube in an incubation series.

Ninhydrin could not be used for detection on the paper, since it causes a variable degree of loss of the α-carboxyl group of glutamic acid which in the present study was radioactive. Fluorescamine is reported to be very much more sensitive than ninhydrin in aqueous solution, but considerable difficulties were experienced in its use for detection on paper, until soaking in borate buffer at pH 10.6 was adopted. Dipping the paper in 3% (v/v) pyridine in acetone (Vandekerckhove & Van Montagu, 1974) before fluorescamine treatment was not effective.

Mechanism of the enzyme reaction

Frère (1973) listed ten possible mechanisms and their rate equations for bimolecular transfer reactions in which hydrolysis of the donor molecule could also occur. In practice, however, the differences between the rate equations are not very great, and the precision of the data in the present case was not sufficient to justify sophisticated model-discrimination tests (Hurst *et al.*, 1973; Mannervik, 1974). Nevertheless, the kinetic and chemical data are on the whole compatible with a non-sequential mechanism with a hydrolytic branch, such as scheme 1:A of

Table 2. *Binding of* [*Glu*-^{14}C]*GSH*/[*Gly*-^{3}*H*]*GSH to enzyme protein*

The substrate solution contained [*Glu*-^{14}C]GSH/[*Gly*-^{3}H]GSH (2.36mM) (each isotope approximately 5μCi/μmol) and dithiothreitol (5mM) in 0.1M-Tris/HCl, pH 8.4. In the control tubes, the enzyme (300μg of protein per ml) was mixed with 2ml of 20% (w/v) ice-cold trichloroacetic acid, and 0.5mg of bovine serum albumin was added, followed by the substrate solution. In the experimental tubes, the enzyme was mixed with substrate solution (the final concentration of GSH was 0.28mM) and incubated at room temperature for 10s; 2ml of ice-cold 20% trichloroacetic acid was then added, followed by 0.5mg of bovine serum albumin. After standing for 30min on ice, each mixture was filtered on a Whatman GF/C disc, followed by four washes with 4ml of 5% trichloroacetic acid. The discs were each soaked in 1ml of 2% (w/v) sodium dodecyl sulphate solution overnight, 10ml of Aquasol was added, and the radioactivity determined with several 80min counting periods.

Incubation mixture	Enzyme (ml)	Substrate solution (ml)	^{3}H radioactivity (d.p.m.)	^{14}C radioactivity (d.p m.)	^{3}H/^{14}C
		0.02*	508000	535000	0.950
		column†			0.875
Control	0.5	0.066	291	300	0.970
	1.0	0.133	827	912	0.907
	1.5	0.200	1251	1341	0.933
Experimental	0.5	0.066	799	2139	0.374
	1.0	0.133	1811	4923	0.370
	1.5	0.200	2448	7513	0.326

* Duplicate samples of the substrate solution were counted directly in Aquasol.

† A sample of the substrate solution was chromatographed on Dowex 1-X2 in the presence of β-mercaptoethanol (Furano, 1971); the isotope ratio given is the mean observed across the eluted GSH peak.

Frère (1973). The rate equations for this mechanism had been worked out earlier by Folk (1969) and by Chung & Folk (1972) (see the Appendix) for the rather similar case of the soluble transglutaminases.

According to this mechanism, the transfer should lead to a set of converging lines for double-reciprocal data plots (eqn. 3). The term $K_{ah}\cdot K_{b}$ in eqn. (3) (see the Appendix) distinguishes it from a simple non-sequential mechanism (which is associated with a set of parallel lines), and it may be predicted that variation of the methionine concentration from 1.0 to 10.0mM should give a detectable change of gradient. It is important to realize that a set of parallel lines is almost impossible to rationalize on theoretical grounds in an enzyme reaction which is unequivocally causing both hydrolysis and transfer simultaneously. The observed parallelism (Fig. 2) may be due partly to insufficient precision of the data and partly to the great disparity in the values of K_{ah} and K_{b}. Tate & Meister (1974*b*) used GSH and [^{14}C]methionine (10–25mM) to measure transfer and obtained parallel sets of lines; this is to be expected, since at these concentrations of methionine the hydrolysis is so slight as to be negligible. Rather similar problems arose in studying methionine inhibition of hydrolysis. One set of data gave a clearly non-competitive inhibition pattern (eqn. 2, see the Appendix), but other experiments could not define the term $A\cdot B/K_{b}$ in eqn. (2).

The observation of isotope exchange in the present work is open to two interpretations. If indeed isotope exchange occurred, then the fact that it led unambiguously to a converging set of lines (Fig. 4) is highly suggestive of a non-sequential mechanism with a hydrolytic branch (eqn. 5, see the Appendix). Unfortunately, it is not possible on the basis of the kinetic data alone to exclude a simple ordered sequential reaction between γ-glutamylmethionine as the donor substrate and [^{14}C]methionine as the acceptor, which would also lead to a set of converging lines.

Previous reports have included the suggestion that the enzyme followed a non-sequential mechanism, on the basis of rather limited kinetic results at very high substrate concentrations, and therefore have concluded that a γ-glutamyl-enzyme should exist (Tate & Meister, 1974*a*,*b*). By using [*Glu*-^{14}C]-GSH/[*Gly*-^{3}H]GSH at a final concentration of 0.28mM (and bearing in mind the K_{ah} value of 13.9μM), it was found (Table 2) that ^{14}C was preferentially bound to the precipitated protein, and that the amount of ^{14}C bound was proportional to the amount of enzyme protein used. This preliminary experiment argues very strongly in favour of the occurrence of a γ-glutamyl enzyme. The hydrolytic capacity of the enzyme may make it very difficult to proceed to isolate γ-glutamyl oligopeptides related to the active site of the enzyme. It is noteworthy that Mizobuchi & Buchanan (1968) found that ice-cold trichloroacetic acid released ^{14}C from what appeared to be a γ-glutamyl enzyme involving a thioester bond. So far as is known, –SH groups are not very important in the catalytic action of γ-glutamyltransferase (see Elce *et al.*, 1974),

so that other types of covalent linkage of the γ-glutamyl group must be considered.

The formation of about 4% of γ-glutamyl-GSH was demonstrated in the absence of methionine in extreme conditions designed to maximize its formation (Table 1). It has been assumed that γ-glutamyl-GSH was not formed to a significant extent during the kinetic experiments reported here. The marked substrate inhibition observed at higher GSH concentrations (Fig. 3) suggests that GSH must then bind at an inhibitory site without leading to significant reaction. Milbauer & Grossowicz (1965), by using a bacterial enzyme and 20mM-GSH, reported the formation of γ-glutamyl-GSH only in the absence of alternative acceptors; Goore & Thompson (1967), who used a plant enzyme, stated without details that γ-glutamyl-GSH was not formed. With *S*-methyl-GSH, Tate & Meister (1974*a*) observed small amounts of γ-glutamyl-*S*-methyl-GSH.

Physiological considerations

It should be acknowledged that the enzyme used in this work was obtained by using detergents, and its behaviour is not necessarily the same as that of the membrane-bound enzyme *in vivo*.

The evidence for involvement of γ-glutamyltransferase in amino acid transport was summarized by Meister (1973, 1974). The enzyme is most abundant in renal tissue, but has also been found in significant amounts in erythrocyte membranes (Palekar *et al*., 1974), in the ciliary body and lens (Ross *et al*., 1973) and in the choroid plexus (Okonkwo *et al*., 1974), where a role in amino acid transport is feasible. By the use of an inhibitor of 5-oxoprolinase, an enzyme subsequent to γ-glutamyltransferase in the γ-glutamyl cycle, it was shown that in the mouse *in vivo* the renal transport of glutamate, glutamine, methionine and valine was associated with an accumulation of 5-oxoproline in the kidney; glycine and lysine did not cause this accumulation (Van der Werf *et al*., 1974). The reservations may be made that the tissue concentrations of amino acids used by Van der Werf *et al*. (1974) were three to eight times the normal physiological values and that the effect of glutamate in increasing 5-oxoproline concentration is not consistent with the usual classification of amino acids in transport (Milne, 1971). The transport *in vivo* appears to be carried out by separate processes for several sub-groups of the amino acids, but *in vitro* most of the common amino acids have 50–100% of the activity of glutamine as acceptors of the γ-glutamyl group of GSH, and have apparent Michaelis constants, with *S*-pyruvoyl-GSH as the donor, of 2–10mM (Tate & Meister, 1974*b*). By using GSH itself, the Michaelis constant of methionine has been found in the present work to be 4.67±0.7mM. In contrast with these values, the concentrations of amino acids in plasma are in the 0.05–0.5mM range (e.g., [methionine] = 68μM in plasma) (Seta *et al*., 1973; Young *et al*., 1973), all much lower than the Michaelis constants, so that *in vivo* the rate of transfer must be very small compared with that of hydrolysis. Fig. 5 shows that the rate of transfer is very low when the concentration of methionine is less than 0.1mM, whereas at a concentration of methionine of 6mM or more [the type of assay condition commonly used previously by many workers (e.g. Tate & Meister, 1974*b*)], the hydrolysis rate is the lower, and rapidly becomes negligible, as the concentration of methionine increases.

Orlowski & Wilk (1975) have suggested that the formation of some γ-glutamyl amino acids and hence of the observed 5-oxoproline may be due largely to the action of the enzyme γ-glutamylcysteine synthetase (EC 6.3.2.2).

In addition, it has been shown that γ-glutamyltransferase is identical with a previously recognized phosphate-independent glutaminase (Curthoys & Kuhlenschmidt, 1975); and the glutaminase distribution indicates that the enzyme is located on the luminal surface of the brush-border membrane of the proximal straight tubule (Kuhlenschmidt & Curthoys, 1975), where it is exposed to the kidney filtrate and not to the interior of the tubule cells.

Although very many reports have appeared on the subject of the GSH concentration in erythrocytes, which is high (Koivusalo & Uotila, 1974), no precise measurement of plasma GSH concentration has been made. Elwyn *et al*. (1968) state that the plasma concentration of GSH is zero; their analytical method is not clearly explained, but it may be deduced from the report by Elwyn (1966) that a plasma concentration of GSH of less than 30μM would not be detected (see 'Note Added in Proof').

In a single patient it was observed that a genetic defect in γ-glutamyltransferase was associated with glutathionemia and glutathionuria, but that amino acid transport appeared to be normal (Schulman *et al*., 1975). In addition to this, at least one of the renal cysteinylglycinase activities appears to be membrane-bound and concentrated in the same region of the kidney tubule as the γ-glutamyltransferase (N. P. Curthoys, personal communication). Together these reports imply that a major function of γ-glutamyltransferase and cysteinylglycinase is to hydrolyse glutathione in the renal filtrate, permitting recovery of its constituent amino acids by the kidney tubule. The very low Michaelis constant of the γ-glutamyltransferase (K_{ah} = 13.9μM) is then consistent with the fact that the plasma GSH concentration, and therefore presumably the GSH concentration in the renal filtrate, is very low.

In conclusion it may be stated that some knowledge has been gained of the kinetic mechanism of the enzyme and of the course of its reaction with GSH and methionine. Its physiological role is not yet certain, but its contribution to amino acid reabsorption would seem to be small, and it is possible that its principal function lies in hydrolysis of GSH in the renal filtrate.

Note Added in Proof (Received 1 December 1975)

We have since discovered the paper by Tietze (1969), in which the GSH + GSSG concentration in rat plasma is reported to be about 5 μM. This material is probably largely in the form of GSSG, and we have no precise information about the Michaelis constant of this enzyme for GSSG.

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APPENDIX

For convenience in discussion of the data, some equations based on the treatment by Folk (1969) are given below. The notation used by Folk (1969) has been largely retained to facilitate reference to his paper; the symbol used by Folk (1969), if different, is given in parentheses. The equations are very similar to those given for 'scheme 1:A' by Frère (1973).

Definitions

A = [^{14}C]GSH concentration; B = [methionine]; P = [Cys-Gly]; Q = [γ-Glu-Met]; K_{ah} = Michaelis constant for GSH in hydrolysis; K_{at} = Michaelis constant for GSH in transfer; $K_b(K_{bt})$ = Michaelis constant for methionine; $V_{max.h}(V_a)$ = maximum velocity of hydrolysis; $V_{max.t}(V_{ab})$ = maximum velocity of transfer; $v_{oh}(v)$ = initial velocity of hydrolysis;

$v_{ot}(v)$ = initial velocity of transfer. All other terms are precisely as defined by Folk (1969).

Hydrolysis of GSH in the absence of methionine:

$$v_{oh} = \frac{V_{max.h} \cdot A}{A + K_{ah}} \quad (1)$$

Hydrolysis of GSH in the presence of methionine:

$$v_{oh} = \frac{V_{max.h} \cdot A}{A + K_{ah} + \frac{K_{ah} \cdot B}{K_{ibb}} + \frac{A \cdot B}{K_b}} \quad (2)$$

Transfer, in the absence of products:

$$v_{ot} = \frac{V_{max.t} \cdot A \cdot B}{A \cdot B + K_b \cdot A + K_{at} \cdot B + K_{ah} \cdot K_b} \quad (3)$$

Transfer in the presence of cysteinylglycine:

$$v_{ot} = \frac{V_{max.t} \cdot A \cdot B}{A \cdot B + K_b \cdot A + K_{at} \cdot B + K_{ah} \cdot K_b + \frac{K_{ia} \cdot K_b \cdot P}{K_{ip}} + \frac{K_b \cdot A \cdot P}{K_{ip}}} \quad (4)$$

Isotope exchange between radioactive methionine (B*) and γ-glutamylmethionine:

$$v_0 \text{ (isotope exchange)} = \frac{V_2^* \cdot B^* \cdot Q}{B^* \cdot Q + K_{iq} \cdot B^* + K_{ib} \cdot Q + K_{ib} \cdot K_{qh}} \quad (5)$$

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